CELL BIOLOGY, ENGINEERING. For the article "Primary cilia mediate mechanosensing in bone cells by a calcium-independent mechanism," by Amanda M. D. Malone, Charles T. Anderson, Padmaja Tummala, Ronald Y. Kwon, Tyler R. Johnston, Tim Stearns, and Christopher R. Jacobs, which appeared in issue 33, August 14, 2007, of *Proc Natl Acad Sci USA* (104:13325–13330; first published August 2, 2007; 10.1073/pnas.0700636104), the authors wish to note the following. "The authors have realized that the concentration of BAPTA used in the experiment shown in Fig. 4*C* is insufficient to fully chelate extracellular calcium. Thus, our conclusion that extracellular calcium entry is not required for calcium flux in MC3T3-E1 cells is invalid. However, our results demonstrating a lack of correlation between primary cilia and calcium flux, calcium flux in cells lacking primary cilia, and calcium flux after treatment with gadolinium chloride support our conclusion that mechanosensation via the primary cilium is independent of calcium flux and polycystin 2 in bone cells. The authors regret any confusion this error has caused."

www.pnas.org/cgi/doi/10.1073/pnas.0711497105

DEVELOPMENTAL BIOLOGY. For the article "An *Ryr1*^{I4895T} mutation abolishes Ca^{2+} release channel function and delays development in homozygous offspring of a mutant mouse line," by Elena Zvaritch, Frederic Depreux, Natasha Kraeva, Ryan E. Loy, Sanjeewa A. Goonasekera, Simona Boncompagi, Alexander Kraev, Anthony O. Gramolini, Robert T. Dirksen, Clara Franzini-Armstrong, Christine E. Seidman, J. G. Seidman, and David H. MacLennan, which appeared in issue 47, November 20, 2007, of *Proc Natl Acad Sci USA* (104:18537–18542; first published November 14, 2007; 10.1073/pnas.0709312104), the authors note that the author name Simona Boncompagi should have appeared as Simona Boncompagni. The online version has been corrected. The corrected author line appears below.

Elena Zvaritch, Frederic Depreux, Natasha Kraeva, Ryan E. Loy, Sanjeewa A. Goonasekera, Simona Boncompagni, Alexander Kraev, Anthony O. Gramolini, Robert T. Dirksen, Clara Franzini-Armstrong, Christine E. Seidman, J. G. Seidman, and David H. MacLennan

www.pnas.org/cgi/doi/10.1073/pnas.0711490105

MEDICAL SCIENCES. For the article "Mechanism of and requirement for estrogen-regulated *MYB* expression in estrogen-receptor-positive breast cancer cells," by Yvette Drabsch, Honor Hugo, Rui Zhang, Dennis H. Dowhan, Yu Rebecca Miao, Alan M. Gewirtz, Simon C. Barry, Robert G. Ramsay, and Thomas J. Gonda, which appeared in issue 34, August 21, 2007, of *Proc Natl Acad Sci USA* (104:13762–13767; first published August 9, 2007; 10.1073/pnas.0700104104), the authors note that on page 13767, left column, in *Myb Knockdown by RNA Interference*, the first sequence in the second sentence, "5′-**GAACCUCUUAC-AAUUUGCAGAAACACU**UUCAUGAGA**AGUGUUUCUGC-AUUGUGUAAGAGGUUC**UU-3′," should instead read: "5′-**GAACCUCUUAGAAUUUGCAGAAACACU**UUCAUGAG-**AAGUGUUUCUGCAAAUUCUAAGAGGUUC**UU-3′. This error does not affect the conclusions of the article.

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Mechanism of and requirement for estrogen-regulated *MYB* expression in estrogen-receptor-positive breast cancer cells

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Edited by Suzanne Cory, The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia, and approved June 29, 2007 (received for review January 7, 2007)

***MYB* (the human ortholog of c-*myb*) is expressed in a high proportion of human breast tumors, and that expression correlates strongly with estrogen receptor (ER) positivity. This may reflect the fact that *MYB* is a target of estrogen/ER signaling. Because in many cases *MYB* expression appears to be regulated by transcriptional attenuation or pausing in the first intron, we first investigated whether this mechanism was involved in estrogen/ER modulation of *MYB*. We found that this was the case and that estrogen acted directly to relieve attenuation due to sequences within the first intron, specifically, a region potentially capable of forming a stem–loop structure in the transcript and an adjacent poly(dT) tract. Secondly, given the involvement of *MYB* in hematopoietic and colon tumors, we also asked whether *MYB* was required for the proliferation of breast cancer cells. We found that proliferation of ER$^+$ but not ER$^-$ breast cancer cell lines was inhibited when *MYB* expression was suppressed by using either antisense oligonucleotides or RNA interference. Our results show that *MYB* is an effector of estrogen/ER signaling and provide demonstration of a functional role of *MYB* in breast cancer.**

antisense | cell cycle | proliferation | short hairpin RNA | transcriptional attenuation

The *MYB* oncogene encodes a transcription factor (Myb) that has predominantly been associated with normal and leukemic hematopoiesis. This association is based on its pattern of expression (1, 2), the consequences of reduced or ablated expression (3, 4), and the ability of activated or overexpressed forms of Myb to contribute to leukemogenesis in several species (reviewed in ref. 5). It has also been known for some time that *MYB* is expressed at relatively high levels in at least two epithelial tumors: colon (6) and breast cancer (7). In the former, data are accumulating that indicate a functional role in carcinogenesis; these include a correlation of expression level with poor prognosis (8) and the presence of mutations that are likely to dysregulate *MYB* expression in colon carcinomas (9, 10).

Guerin *et al.* (7) first reported that *MYB* was expressed in a high proportion of human breast tumors and that expression correlated strongly with estrogen receptor (ER) positivity. This has been borne out by other studies that have examined cell lines (e.g., ref. 11) and by analysis of data from studies of large numbers of tumors by using microarray expression profiling [see supporting information (SI) Fig. 6].

Moreover, a basis for the correlation is suggested by reports showing that estrogen/ER signaling directly modulates *MYB* expression (11, 12). Interestingly, one study (11) suggests that ER signaling enhances *MYB* expression at a level other than transcriptional initiation. In many cell types, *MYB* expression appears to be regulated by transcriptional attenuation or pausing in the first intron (13, 14), and our work has shown that this involves a region potentially capable of forming a stem–loop (SL) structure in the transcript and an adjacent poly(dT) region (9, 10). Intriguingly, this motif is frequently mutated in colon carcinomas but not in breast cancer (10), suggesting that another mechanism is responsible for overcoming attenuation in breast cancer cells. Also in contrast to colon cancer, there are essentially no data that address a functional role for *MYB* in breast carcinogenesis, with the exception of a correlation between *BRCA1* mutation and *MYB* amplification in ≈30% of *BRCA1* mutant tumors (15).

Here, we report that estrogen/ER acts directly to relieve transcriptional attenuation due to sequences within the first intron, specifically, the SL–poly(dT) motif. Moreover, given the involvement of *MYB* in hematopoietic and colon tumors and that *MYB* is a direct ER target, we asked whether *MYB* was required for the proliferation and/or survival of ER$^+$ breast cancer cells. By using antisense (AS) oligonucleotides and RNA interference to block *MYB* expression, we found that this was indeed the case, providing functional evidence of a role for *MYB* in breast cancer.

Results

Transcriptional Regulation of *MYB* Expression by Estrogen. To confirm *MYB* regulation by estrogen, we used the well characterized ER$^+$ breast cancer cell line MCF-7. Fig. 1*A* shows that β-estradiol treatment resulted in a rapid 4- to 5-fold increase in expression determined by quantitative RT-PCR and that induction of *MYB* did not require protein synthesis because it was insensitive to treatment of the cells with cycloheximide, which is in agreement with other studies (12). The kinetics of induction were very similar to those of another, well documented ER target, pS2 (Fig. 1*A*). We also confirmed (*i*) a concomitant increase in the rate of proliferation of MCF-7 cells (data not shown; see also Fig. 3); (*ii*) that ER$^-$ breast tumor cells (MDA-MB-468) expressed 10- to 40-fold less *MYB* mRNA than MCF-7

Author contributions: H.H., Y.R.M., R.G.R., and T.J.G. designed research; Y.D., R.Z., and D.H.D. performed research; Y.D., H.H., R.Z., Y.R.M., A.M.G., S.C.B., and R.G.R. contributed new reagents/analytic tools; Y.D., R.G.R., and T.J.G. analyzed data; and T.J.G. wrote the paper.

The authors declare no conflict of interest.

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Abbreviations: AS, antisense; CAT, chloramphenicol acetyl transferase; Dox, doxycycline; ER, estrogen receptor; SCR, scrambled control; sh, short hairpin; SL, stem–loop.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0700104104/DC1.

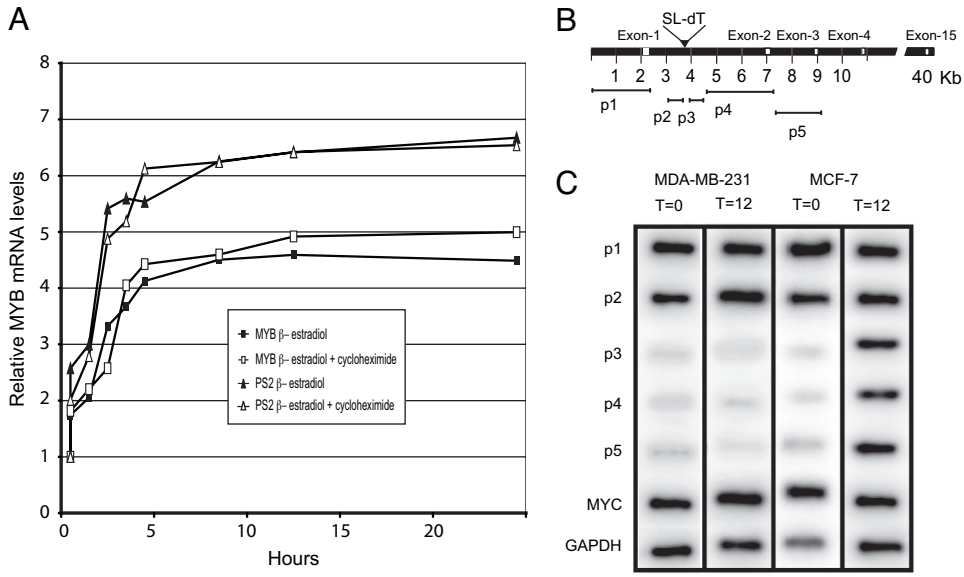

Fig. 1. Estrogen induces increased *MYB* mRNA levels and overcomes transcriptional attenuation. (*A*) MCF-7 cells were treated with 10 nM β-estradiol for the indicated times in the presence or absence of 10 μg/ml cycloheximide, and the levels of *MYB* and *pS2* mRNA were quantitated by quantitative RT-PCR. (*B*) Schematic representation of part of the human *MYB* gene illustrating the location of the SL and poly(dT) motifs (SL-dT) in the first intron and the location of probes (p1–p5) used in nuclear run-on transcription assays. (*C*) Transcriptional activity, measured by nuclear run-on assays, in the 5′ region of the *MYB* gene was compared before ($T = 0$) and after ($T = 24$) exposure of MCF-7 and MDA-MB-231 cells to 10 nM β-estradiol for 12 h. The α^{32}P-UTP-labeled RNA transcripts were hybridized to immobilized cRNA probes as indicated and detected by using a PhosphorImager.

cells; and (*iii*) that this did not increase in response to β-estradiol (SI Fig. 7).

In view of studies showing that *MYB* expression is frequently regulated by transcriptional pausing within intron 1 (9, 13, 14), we next performed nuclear run-on assays by using labeled RNA from ER^+ (MCF-7) and ER^- (MDA-MB-231) breast cancer cells. Fig. 1*C* shows that in MDA-MB-231 cells and in untreated MCF-7 cells, transcription could be detected in all cases by probes (p1 and p2; Fig. 1*B*) spanning exon 1 and the first ≈1 kb of intron 1. However, virtually no transcription proceeding through the first intron past the SL–poly(dT) motif to exons 2 and 3 could be detected. In contrast, in β-estradiol-treated MCF-7 cells, the level of transcripts hybridizing to probes encompassing the 3′ portion of intron 1, and exons 2 and 3 (probes p3–p5; Fig. 1*B*), increased strikingly (13-, 63-, and 109-fold, respectively; see SI Fig. 8 for quantitation). These data imply that estrogen/ER signaling relieved a block of transcriptional elongation in intron 1 and that this block occurred within the vicinity of the SL–poly(dT) motif.

To characterize the requirements for transcriptional attenuation and its relief by β-estradiol, we next used a chloramphenicol acetyl transferase (CAT) reporter construct containing the *MYB* promoter, exon 1, and roughly the first half of intron 1, including the SL–poly(dT) motif (Fig. 2*A*) (10). This reporter was transfected into MCF-7 cells, which were cultured in the absence or presence of β-estradiol. Fig. 2*B* shows that CAT activity was increased 3.5-fold in the presence of estrogen, indicating that all of the elements required for β-estradiol

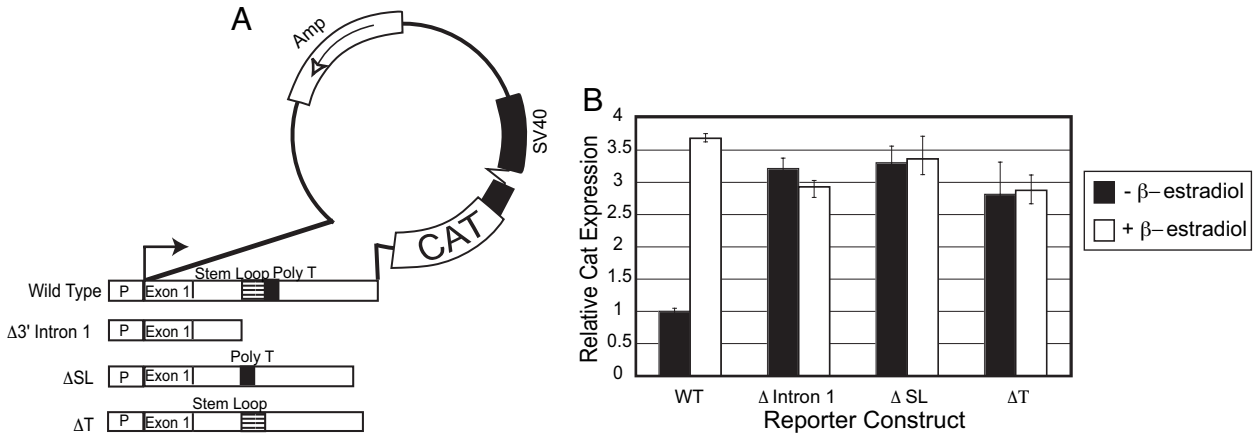

Fig. 2. Reporter assays reveal that β-estradiol relieves the suppression of transcription due to the SL–poly(dT) motif. (*A*) Schematic illustration of the *MYB* reporter construct in which CAT transcription is driven by the human *MYB* promoter through exon 1 and part of intron 1 (WT). Also shown are deletion constructs in which the entire SL–poly(dT) motif is deleted along with the remaining 3′ sequences of intron 1 (Δ3′ intron 1), only the SL motif is deleted (ΔSL), or only the poly(dT) motif is deleted (ΔT). (*B*) CAT activity (± SEM) in MCF-7 cells transiently transfected with the reporter constructs shown in *A* and cultured in the presence or absence, as indicated, of 10 nM β-estradiol for 12 h.

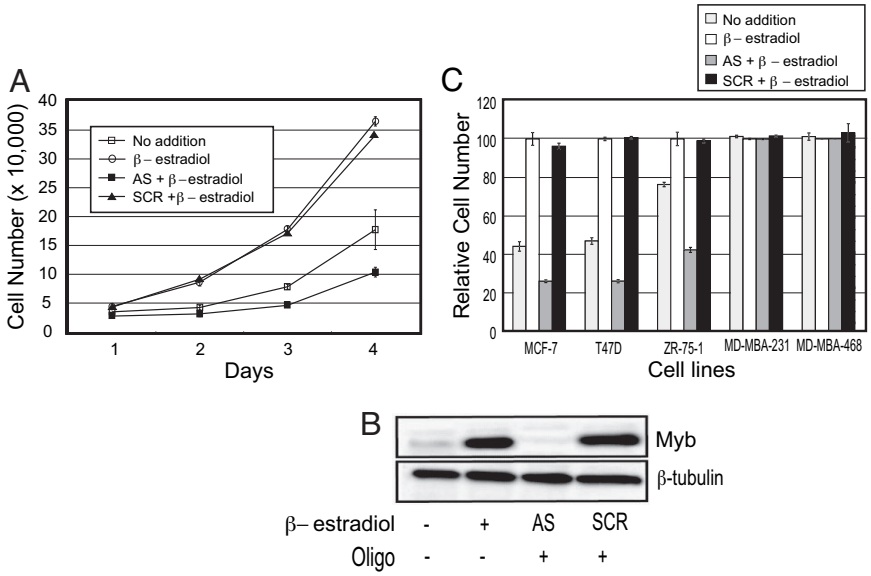

Fig. 3. AS *MYB* oligonucleotide inhibits the proliferation of ER$^+$ but not ER$^-$ breast cancer cell lines. (*A*) Growth of MCF-7 cells cultured in the absence (no addition) or presence of 10 nM β-estradiol or cultured in the presence of β-estradiol and also treated with either 1 μM SCR or 1 μM *MYB* AS oligonucleotides. Mean cell numbers (± SD) of triplicate samples were determined daily as shown. (*B*) AS *MYB* oligonucleotide, but not the SCR oligonucleotide, decreases Myb protein levels in MCF-7 cells. Total cell lysates were collected for Western blot analysis with Myb and β-tubulin (as a loading control) antibodies. (*C*) The *MYB* AS oligonucleotide inhibits proliferation of ER$^+$ but not ER$^-$ breast cancer cell lines. The indicated cell lines were treated as in *A*, and the cell number was determined after 3 days. The results (± SEM) normalized to the cell numbers of β-estradiol-only-treated cultures of each cell line (set at 100%) are shown.

responsiveness are contained within the *MYB* sequences present in the reporter construct. Next, to examine the roles of the SL and poly(dT) motifs, these were deleted from the reporter construct either singly or together. Each of these constructs showed the same level of activity in the absence and presence of β-estradiol, and, critically, this level of activity was very similar to that seen in the parental construct in the presence of β-estradiol (Fig. 2*B*). Thus, β-estradiol appeared to relieve a block to expression that normally requires both the SL and poly(dT) motifs.

Requirement for *MYB* for Estrogen-Dependent Proliferation. The observations described above indicate that *MYB* expression in the ER$^+$ breast cancer cell line MCF-7 is regulated by β-estradiol/ER signaling, leading to the relief of transcriptional attenuation within intron 1. However, what are the implications of this for ER$^+$ breast cancer? To address this question, we treated MCF-7 cells with a previously validated *MYB* AS oligonucleotide (16). Fig. 3*A* shows that the *MYB* AS oligonucleotide, but not a scrambled control (SCR) oligonucleotide, strongly inhibited β-estradiol-dependent proliferation of these cells. Specific knockdown of Myb by the AS oligonucleotide was shown by Western blotting (Fig. 3*B*), which also showed that, as expected, Myb was induced by β-estradiol and that the SCR oligonucleotide had no effect on Myb levels. Quantitative RT-PCR confirmed specific knockdown of c-*MYB*, but not B-*MYB*, mRNA (SI Fig. 9*A*). We noted that growth of AS-treated cells was slower than that of cells not treated with β-estradiol (Fig. 3*A*), probably because of further suppression of the low levels of *MYB* present in such cells. AS oligonucleotide treatment had no effect on cell viability (data not shown), indicating that the effect was on proliferation *per se*.

To confirm the generality of this observation, we treated several other breast cancer cell lines with the *MYB* AS oligonucleotide. As with MCF-7 cells, the proliferation of ER$^+$ T47D and ZR-75-1 cells in the presence of β-estradiol was strongly inhibited by the AS, but not the SCR oligonucleotides; however, there was no effect on the proliferation of the ER$^-$ MDA-MB-231 or MDA-MB-468 cell lines under any of the conditions (Fig. 3*C*). Knockdown of *MYB* was confirmed by quantitative RT-PCR in each case (SI Fig. 9*B*). Thus, it appears that *MYB* is necessary for the proliferation of ER$^+$ but not ER$^-$ breast cancer cell lines, which is consistent with the relative expression of *MYB* in these two classes. Because it is possible that inhibition by AS oligonucleotides was influenced by nonspecific or off-target effects, we also used a second approach, RNA interference, to stably knockdown Myb expression. An inducible short hairpin (sh)RNA-expressing lentiviral vector, pLVTSH, was used to stably transduce MCF-7 cells. Briefly, this vector (Fig. 4*A* and data not shown) expresses shRNA under the control of a Tet-repressor-regulated H1 promoter and also encodes the Tet repressor itself, as well as GFP. Thus, shRNA is expressed in transduced cells only in the presence of tetracycline analogs. Fig. 4 *B* and *C* shows that proliferation of MCF-7 cells transduced with a *MYB* shRNA vector was inhibited only in the presence of doxycycline (Dox). Cells transduced with a control shRNA vector or empty pLVTSH showed no reduction in growth under either condition. Specific knockdown of Myb was confirmed by Western blotting (Fig. 4*D*).

Inhibition of proliferation could reflect either cell death or a block in cell cycle progression. The former was ruled out by studies on cell viability and apoptosis induction (data not shown). DNA content analysis (Fig. 5) revealed that induction of *MYB* shRNA resulted in a block of progression from the G_1/S and G_2/M phases, consistent with previous reports in other cell types (e.g., ref. 17).

Discussion

Estrogen Regulation of MYB Expression. Although previous studies have shown that *MYB* is a target of estrogen/ER signaling, the precise mechanism involved has not been determined. The data of Gudas *et al.* (11) suggest that regulation is not at the level of transcriptional initiation according to nuclear run-on studies that used a large cDNA fragment as a probe. However, the use of such a probe would not necessarily reveal an attenuation mechanism such as that reported for *MYB* in other cell systems, whereby transcriptional elongation through the first intron is

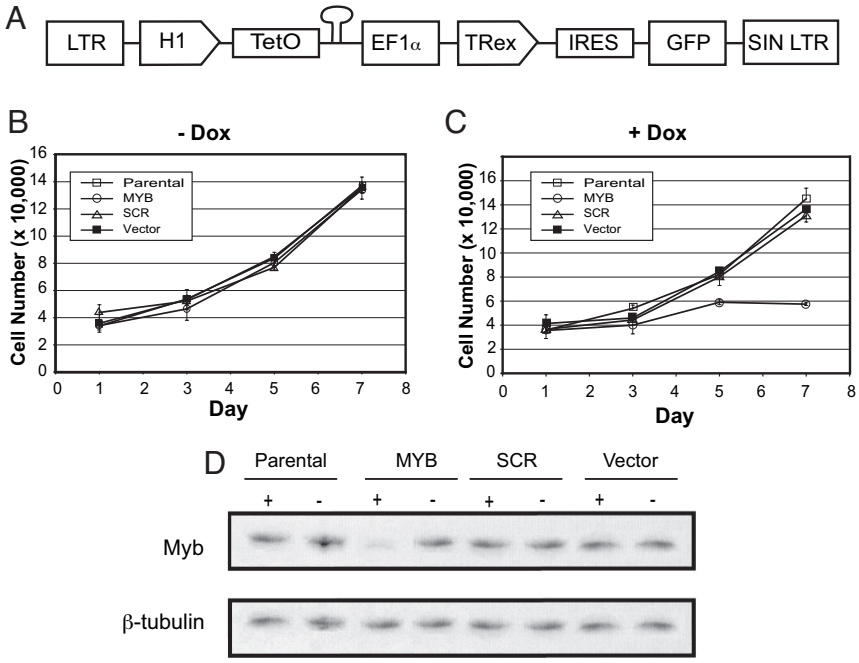

Fig. 4. Knockdown of *MYB* by inducible shRNA decreases MCF-7 proliferation and Myb protein expression. (*A*) Schematic representation of the lentiviral vector pLVTSH showing the following key elements (left to right): LTR, H1 promoter, Tet operator, shRNA template, EF1α promoter, Tet repressor, internal ribosome entry site, GFP, self-inactivating LTR. (*B* and *C*) Parental MCF-7 cells or cells stably transduced with pLVTSH were grown in 10 nM β-estradiol in the absence or presence of Dox over 7 days. Neither the empty vector (Vector) nor a vector expressing a SCR affected proliferation, but the *MYB* shRNA vector inhibited proliferation when cells were grown in the presence of Dox. (*D*) Western blot analysis showing the decreased expression of Myb in only the *MYB* shRNA vector-transduced cells after treatment with Dox for 24 h. The membrane was also probed for β-tubulin as a loading control.

regulated to allow or block the production of full-length *MYB* transcripts (9, 13, 14). The present study has shown that estrogen/ER signaling acts to relieve this block to transcriptional elongation within the first intron. Thus, transcriptional attenuation of *MYB* in breast cancer cells appears similar to that described in other cell types, such as hematopoietic cells (13, 14) and colon carcinoma (9, 10). Attenuation appears to occur within a region between 1.4 kbp and 2.2 kbp from the start of intron 1 which includes the SL–poly(dT) motif, and indeed our nuclear run-on and reporter studies support the notion that this motif represents the site of transcriptional blockage.

A potential artifact has been reported in nuclear run-on studies on transcription of the *MYC* gene, whereby transcription can appear to pause within, at most, a few hundred base pairs of the promoter because of release of stalled RNA polymerase complexes (18). However it is very unlikely that such an artifact can account for our results, because, as also discussed by Hugo *et al.* (10), (*i*) run-on experiments indicated that *MYB* transcription was blocked in most cell types >1 kbp from the transcription start site, and (*ii*) the SL and poly(dT) sequences also affected transcription of a reporter construct (i.e., in studies not involving nuclear run-on assays).

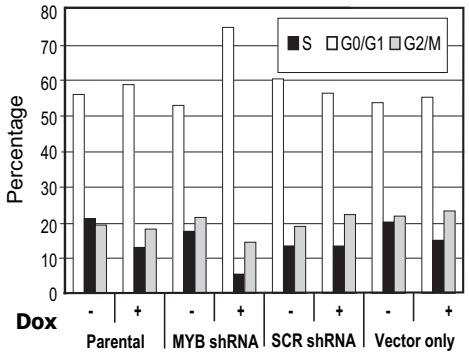

Fig. 5. Knockdown of *MYB* with shRNA blocks cell cycle progression from the G_1/S phase in MCF-7 cells. The cell lines used in the studies illustrated in Fig. 4 were grown in the presence of β-estradiol with or without Dox, as indicated, for 72 h. They were then harvested for cell cycle distribution analysis by propidium iodide staining and flow cytometry; percentages of cells in G_0/G_1, S, and G_2/M are shown for each treatment group.

The precise mechanism by which elongation of *MYB* transcripts is blocked by the SL–poly(dT) motif and how this block is overcome has not been resolved, although the involvement of NF-κB family members has been suggested (19). Our finding that the well studied estrogen-/ER-signaling system can modulate this process may shed some light on this issue. Because *MYB* induction by β-estradiol was direct and the elements required were present in the reporter construct, it seemed likely that ER would bind directly to those sequences. In fact, Carroll *et al.* (20) have carried out a global analysis of ER-binding sites in MCF-7 cells and have found that ER does indeed bind to several regions in and around the *MYB* gene, including a site within a ≈600-bp region, present within our reporter construct, immediately upstream of the SL–poly(dT) motif (see http://research.dfci.harvard.edu/brownlab/datasets/index.php). We have confirmed this observation by chromatin immunoprecipitation, which showed β-estradiol-induced binding of ER to a region close to the SL–poly(dT) motif (SI Fig. 10). Inspection of this region revealed two potential ER-binding sites. However, mutation of these motifs failed to affect the β-estradiol responsiveness of the reporter construct (data not shown), implying that ER either binds to a noncanonical sequence or that it interacts via another transcription factor bound within this region. Given the location of the ER-binding site near the presumptive attenuation region, it is intriguing that the cyclin T1 subunit of the positive transcription elongation factor b complex, which is known to promote transcription elongation (reviewed in ref. 21), has recently

been reported to interact directly with the ER (22). Moreover this factor is an important mediator of relief of the transactivation-responsive-region-mediated block to elongation of HIV RNA by HIV Tat (reviewed in ref. 23); interestingly, the transactivation-responsive region is known to form a SL structure similar to that predicted for the SL element in *MYB*. It may be useful to explore the involvement of positive transcription elongation factor b components in the action of β-estradiol on *MYB* expression.

Requirement for *MYB* in ER$^+$ Breast Cancer. To the best of our knowledge, this report is the first to demonstrate a functional role for *MYB* in breast cancer. We have shown that inhibition of *MYB* expression severely impairs the proliferation of ER$^+$, but not ER$^-$, breast cancer cell lines. Thus it is likely that the requirement of ER$^+$ tumor cells for *MYB*, and the regulation of *MYB* by estrogen/ER signaling together underlie the strong correlation between ER and *MYB* expression in primary breast tumors (see Introduction). The relationship between *MYB* and ER is also consistent with preliminary data indicating expression of c-*myb* in normal, ER$^+$ murine mammary ductal epithelial cells (data not shown). Together, these observations suggest that *MYB* is an important, and possibly essential, effector of estrogen action in breast cancer.

Our findings raise a number of important questions. First, what are the *MYB* target genes that are important for breast cancer cell proliferation? Candidates among known *MYB* targets include *MYC* (24, 25) and *CCNA1* (26), although other regulators of these genes have been identified in breast cancer (e.g., ref. 27). Indeed, *MYC* is itself a direct target of ER signaling (28); therefore, it would seem unlikely that inhibition of *MYB* exerts its effects via *MYC*. Because *MYB* shRNA blocked cell cycle progression at G_1/S, other regulators active at this point in the cell cycle would be logical candidates for further study. Second, is *MYB* expression required for the initiation or progression of mammary tumors, and, third, can enforced *MYB* expression contribute to mammary tumorigenesis? The only reported suggestion of the latter is from the amplification and overexpression of *MYB* in a subset of *BRCA1*-mutant tumors, and very rarely in sporadic tumors (15). The generation of mammary-specific transgenic and gene-targeted mice may help to clarify these issues. Fourth, is there any potential therapeutic benefit in targeting *MYB* in breast cancer? Given the availability of effective drugs that target estrogen/ER signaling, it would seem unlikely that targeting *MYB*, which, in principle, is more difficult, would be a practical first-line therapy. However, resistance to antiestrogens such as tamoxifen is a significant problem; therefore, targeting ER effectors like *MYB* may be beneficial in such cases. Moreover, tamoxifen also induces *MYB* (ref. 29 and data not shown); therefore, it is possible that antiestrogens and potential anti-*MYB* therapies (reviewed in ref. 30) would be effective in combination.

Materials and Methods

Cell Culture. MDA-MB-231, MDA-MB-468, ZR-75-1, and T47D cells were grown routinely in DMEM, 10% FCS, and penicillin–streptomycin–L-glutamine (all from GIBCO/BRL, Grand Island, NY). MCF-7 cells were grown routinely in the same medium supplemented with 1 mM sodium pyruvate/0.1 mM nonessential amino acids/10 μg/ml insulin (Sigma, St. Louis, MO). All cells were grown as a monolayer in 95% air/5% CO_2. For estrogen deprivation, cells were grown in the same media but containing phenol-red-free DMEM and charcoal-stripped FCS for 72 h. Cell proliferation was determined by cell counting by using a Z1 Coulter counter (Beckman Coulter, Sydney, Australia). Cells were plated in 24-well plates at an initial concentration of 5×10^4 cells per well in 0.5 ml of phenol-red-free media overnight and then grown further in the presence or absence of 10 nM 17-β-estradiol (Sigma). Where indicated, cells were exposed to 10 μg/ml cycloheximide (Sigma) for 30 min before the addition of media with or without 10 nM 17-β-estradiol.

Quantitative RT-PCR. Total RNA was prepared by using a RNeasy MiniKit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. One microgram of total RNA was reverse transcribed in a total of 20 μl by using SuperScript III (Invitrogen, Carlsbad, CA). The resulting cDNA was then diluted to a total volume of 100 μl with sterile H_2O. Each real-time PCR consisted of 1 μl of diluted reverse transcriptase product, iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), and 50 nM forward and reverse primers (see below). Reactions were carried out on a RotorGene 3000 (Corbett Research, Sydney, Australia) at 95°C for 10 min, followed by 40 cycles of 95°C for 20 s, 56°C for 20 s, and 72°C for 30 s. Fluorescence measurements analyzed by using the RotorGene 3000 software. The fold-change expression of each gene was calculated by using the ΔΔCT method, with cyclophilin A as an internal control. Primers used for real-time PCR were: c-MYB forward, 5′-GCC AAT TAT CTC CCG AAT CGA-3′; c-MYB reverse, 5′-ACC AAC GTT TCG GAC CGT A-3′; B-MYB forward, 5′-GCC ACT TCC CTA ACC GCA c-3′; B-MYB reverse, 5′-ccc TTG ACA AGG TCT GGA TTC A-3′; cyclophilin A forward, 5′-GGC AAA TGC TGG ACC CAA CAC AAA-3′; cyclophilin A reverse, 5′-CTA GGC ATG GGA GGG AAC AAG GAA-3′.

Nuclear Run-On Transcription Analysis. MCF-7 and MDA-MB-231 were grown in the presence or absence of 10 nM 17-β-estradiol for 24 h before the preparation of nuclei, labeling with ^{32}P-α-UTP, and RNA extraction, all of which was carried out as described previously (9, 10). Probes p1 (pIE2), p4 (pIBg3′), p5 (pIBg2–5′), MYC and GAPDH have been described previously (9). Two additional probes for use in nuclear run on, p2 and p3 (see Fig. 1*B*), were generated with PCR by using *Pfu* polymerase (Invitrogen) and 50 nM p2 forward primer 5′-ATC GAG GAG AAA GAA TTC GAA GAG GGA GAG GAG GAG GA-3′ and reverse primer 5′-AGC TAG CAG ACA GGA TCC AAG GAA AGG CGA ATG GAT TT-3′ or p3 forward primer 5′-AGC TGT GAG AAA GAA TTC TTG CAC ATC TTT GCC TCT G-3′ and reverse primer 5′-ATC TAT GAG AAA GCG GCC GCG GAA CTC CTT GGA AAG ACC-3′, respectively. The products were digested with EcoRI and BamHI or EcoRI and NotI, respectively, and cloned into pBluescript KS+ (Fermentas, Hanover, MD) for the preparation of cRNA probes, which were used for hybridization to labeled nuclear RNA (9, 10). The filters were imaged by using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and the relative intensity of the radioactive bands was measured by using ImageQuant 5.2 (Molecular Dynamics) and normalized to the GAPDH probe.

Reporter Assays. The CAT reporter constructs containing the human c-*MYB* promoter, exon 1, and intron 1 sequences have been described previously (10). MCF-7 cells were transiently transfected with 900 ng of CAT reporter construct plus 100 ng of a β-actin-promoter-driven luciferase expression vector (a gift from C. Popa, University of Queensland, Queensland, Australia) by using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. After transfection, the cells were cultured with or without 10 nM 17-β-estradiol for 12 h before harvesting. CAT expression was assayed with a CAT ELISA kit (Roche, Indianapolis, IN) according to the specifications of the supplier. CAT activity was normalized with respect to luciferase expression, which was determined by using LucLite (PerkinElmer, Waltham, MA) according to the protocol of the manufacturer.

AS Oligonucleotides. c-*MYB* AS (5′-acagaccaacgtttcggaccgtatttctgt-3′) and SCR phosphorothioate oligonucleotides (5′-ctttcgaatgtgacatttcgacacgccagt-3′) were manufactured (University of Pennsylvania, Philadelphia) as published (16). Cells were plated in 24-well plates at an initial concentration of 5×10^4 cells per well overnight and transfected with AS oligonucleotides as described (31). Cell proliferation was determined as described above; experiments were conducted in triplicate and repeated at least twice. Cells were harvested for protein or RNA 24 h after initial transfection.

Western Blotting. Western blot analysis was conducted essentially as described previously (32). Briefly, extracts prepared in SDS loading buffer were resolved in SDS/10% PAGE gels and transferred to PVDF membranes. These were incubated overnight in the presence of anti-c-Myb antibody 1.1 (33) and were developed by using ECL Western blotting substrates (Pierce Biotechnology, Rockford, IL).

Myb Knockdown by RNA Interference. The tetracycline-inducible lentiviral shRNA vector pLVTSH is outlined briefly here. Vectors used encoded a *MYB* shRNA 5′-**GAACCUCUUACAAUUUGCAGAAACACU**UUCAUGAGA**AGUGUUUCUGCAUUGUGUAAGAGGUUC**UU-3′ (with the bold sequences corresponding to sense and AS c-*MYB* mRNA, respectively) and a SCR shRNA 5′-AUAAGAAUGUCCAUCAGUUACGCCAUAUUCAUGAGAUAUGGCGUAACUGAUGGACAUUCUUAUUU-3′. Lentivirus was generated by cotransfection of HEK293T cells with packaging plasmids essentially as described by Barry *et al.* (34) by using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. The supernatant was collected after 48 h and filtered through a 0.45-μm filter before being used to transduce MCF-7 cells. MCF-7 cells were FACS sorted based on expression of pLVTSH-encoded GFP. Proliferation was assayed in triplicate cultures as described above in medium containing 10 nM 17-β-estradiol and/or 5 μg/ml Dox (Sigma, Sydney, Australia). A sample of each culture was collected after 24 h to determine knockdown efficiency by Western blotting.

Cell Cycle Analysis. Cells were harvested, washed twice with ice-cold PBS, and fixed in 70% ethanol at −20°C for at least 30 min. The fixed cells were then washed twice with ice-cold PBS and stained with 50 μg/ml propidium iodide in the presence of 100 μg/ml RNase A for 30 min. Cell cycle distribution was analyzed by using FACS Calibur (Becton Dickinson, San Jose, CA), and the resultant data were analyzed by using CellQuest software (Becton Dickinson).

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1. Westin EH, Gallo RC, Arya SK, Eva A, Souza LM, Baluda MA, Aaronson SA, Wong-Staal F (1982) *Proc Natl Acad Sci USA* 79:2194–2198.
2. Gonda TJ, Sheiness DK, Bishop JM (1982) *Mol Cell Biol* 2:617–624.
3. Mucenski ML, McLain K, Kier AB, Swerdlow SH, Schreiner CM, Miller TA, Pietryga DW, Scott WJ, Jr, Potter SS (1991) *Cell* 65:677–689.
4. Anfossi G, Gewirtz AM, Calabretta B (1989) *Proc Natl Acad Sci USA* 86:3379–3383.
5. Ganter B, Lipsick JS (1999) *Adv Cancer Res* 76:21–60.
6. Torelli G, Venturelli D, Colo A, Zanni C, Selleri L, Moretti L, Calabretta B, Torelli U (1987) *Cancer Res* 47:5266–5269.
7. Guerin M, Sheng ZM, Andrieu N, Riou G (1990) *Oncogene* 5:131–135.
8. Biroccio A, Benassi B, D'Agnano I, D'Angelo C, Buglioni S, Mottolese M, Ricciotti A, Citro G, Cosimelli M, Ramsay RG, *et al.* (2001) *Am J Pathol* 158:1289–1299.
9. Thompson MA, Flegg R, Westin EH, Ramsay RG (1997) *Oncogene* 14:1715–1723.
10. Hugo H, Cures A, Suraweera N, Drabsch Y, Purcell D, Mantamadiotis T, Phillips W, Dobrovic A, Zupi G, Gonda TJ, *et al.* (2006) *Genes Chromosomes Cancer* 45:1143–1154.
11. Gudas JM, Klein RC, Oka M, Cowan KH (1995) *Clin Cancer Res* 1:235–243.
12. Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS (2003) *Endocrinology* 144:4562–4574.
13. Bender TP, Thompson CB, Kuehl WM (1987) *Science* 237:1473–1476.
14. Watson RJ (1988) *Oncogene* 2:267–272.
15. Kauraniemi P, Hedenfalk I, Persson K, Duggan DJ, Tanner M, Johannsson O, Olsson H, Trent JM, Isola J, Borg A (2000) *Cancer Res* 60:5323–5328.
16. Opalinska JB, Kalota A, Gifford LK, Lu P, Jen KY, Pradeepkumar PI, Barman J, Kim TK, Swider CR, Chattopadhyaya J, *et al.* (2004) *Nucleic Acids Res* 32:5791–5799.
17. Gewirtz AM, Anfossi G, Venturelli D, Valpreda S, Sims R, Calabretta B (1989) *Science* 245:180–183.
18. Eick D, Kohlhuber F, Wolf DA, Strobl LJ (1994) *Anal Biochem* 218:347–351.
19. Toth CR, Hostutler RF, Baldwin AS, Jr, Bender TP (1995) *J Biol Chem* 270:7661–7671.
20. Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, Brodsky AS, Keeton EK, Fertuck KC, Hall GF, *et al.* (2006) *Nat Genet* 38:1289–1297.
21. Garriga J, Grana X (2004) *Gene* 337:15–23.
22. Wittmann BM, Fujinaga K, Deng H, Ogba N, Montano MM (2005) *Oncogene* 24:5576–5588.
23. Brigati C, Giacca M, Noonan DM, Albini A (2003) *FEMS Microbiol Lett* 220:57–65.
24. Schmidt CA, Nazarov V, Stevens L, Watson R, Wolff L (2000) *Mol Cell Biol* 20:1970–1981.
25. Nakagoshi H, Kanei Ishii C, Sawazaki T, Mizuguchi G, Ishii S, Kanei-Ishii C (1992) *Oncogene* 7:1233–1240.
26. Muller C, Yang R, Idos G, Tidow N, Diederichs S, Koch OM, Verbeek W, Bender TP, Koeffler HP (1999) *Blood* 94:4255–4262.
27. Coletta RD, Christensen K, Reichenberger KJ, Lamb J, Micomonaco D, Huang L, Wolf DM, Muller-Tidow C, Golub TR, Kawakami K, *et al.* (2004) *Proc Natl Acad Sci USA* 101:6478–6483.
28. Dubik D, Shiu RP (1992) *Oncogene* 7:1587–1594.
29. Hodges LC, Cook JD, Lobenhofer EK, Li L, Bennett L, Bushel PR, Aldaz CM, Afshari CA, Walker CL (2003) *Mol Cancer Res* 1:300–311.
30. Ramsay RG, Barton AL, Gonda TJ (2003) *Expert Opin Ther Targets* 7:235–248.
31. Carroll JS, Swarbrick A, Musgrove EA, Sutherland RL (2002) *Cancer Res* 62:3126–3131.
32. Sambrook J, Frtisch EF, Maniatis T (1989) *Molecular Cloning, a Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, New York).
33. Ramsay RG, Ishii S, Nishina Y, Soe G, Gonda TJ (1989) *Oncogene Res* 4:259–269.
34. Barry SC, Harder B, Brzezinski M, Flint LY, Seppen J, Osborne WR (2001) *Hum Gene Ther* 12:1103–1108.